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<b>(21) International Application Number:</b> PCT/NL96/00278 <b>(22) International Filing Date:</b> 5 July 1996 (05.07.96) <b>(30) Priority Data:</b> 95201871.1 7 July 1995 (07.07.95) EP <b>(34) Countries for which the regional or international application was filed:</b> NL et al. <b>(71) Applicant (for all designated States except US):</b> GENENCOR INTERNATIONAL B.V. [NL/NL]; Wateringseweg 1, NL-2600 MA Delft (NL). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> QUAX, Wilhelmus, Johannes [NL/NL]; Jan Van Galenlaan 8, NL-2253 VB Voorschoten (NL). KERKMAN, Richard [NL/NL]; Koninginneweg 12, NL-2042 Zandvoort (NL). BROEKHUIZEN, Cornelis, Petrus [NL/NL]; Waldeck Pyrmontlaan 25, NL-2281 VK Rijswijk (NL). <b>(74) Agent:</b> SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> NOVEL SECRETION FACTORS FOR GRAM-POSITIVE MICROORGANISMS, GENES ENCODING THEM AND METHODS OF USING IT			
<b>(57) Abstract</b>  Heterologous protein secretion from Gram-positive bacteria, in particular from Bacilli has, with few exceptions, met with little success. Incompatibility of the heterologous proteins with the protein secretion machinery of the host is the main cause of this effect. This limiting factor for the production of heterologous proteins in commercially significant concentrations from Bacillus subtilis is removed by overexpressing the Bacillus subtilis protein FtsY or FtsY protein in combination with overexpression of other members of the bacterial signal recognition particle. Said gene(s) is(are) overexpressed in Bacillus host cells expressing a heterologous protein which then shows an increased amount of the heterologous protein secreted in the surrounding medium.			

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Title: Novel secretion factors for Gram-positive microorganisms, genes encoding them and methods of using it.

This invention relates to processes for the production of proteins by micro-organisms. Specifically, it relates to the secretion of heterologous proteins by micro-organisms, in particular by Gram-positive bacteria, especially by the bacterial host *Bacillus*.

It also relates to (the overexpression of) a novel gene encoding a protein involved in the early stages of prokaryotic protein secretion. Specifically, it relates to the overexpression of said gene within a *Bacillus* host (over)expressing heterologous proteins.

*B. subtilis* and (closely) related bacilli secrete proteins directly into the growth medium to high concentrations. Secretion as a mode of production of proteins of interest, be it homologous to the host or heterologous to the host, be it of recombinant origin or not, provides several advantages over intracellular production. It for instance facilitates purification of the product, it theoretically will lead to a higher yield, no aggregation of the product will occur, and it gives the possibility for continuous cultivation and production. However, attempts to secrete heterologous proteins from *B. subtilis* and (closely) related organisms at commercially significant concentrations have, with few exceptions, met with little success.

Nearly all secreted proteins use an amino-terminal protein extension, known as the signalpeptide, which plays a crucial role in the targeting to, and translocation of precursor proteins across the membrane and which is proteolytically removed by a signalpeptidase during or immediately following membrane transfer. The newly synthesized precursor proteins are recognized by specific proteins in the cytoplasm collectively called chaperones. These chaperones prevent polypeptides, destined for

translocation, to aggregate or fold prematurely leading to an export incompatible conformation.

For instance, SecB, GroEL/GroES and DnaK/DnaJ are the presently known chaperones in the export pathway of E. coli.

5 For the productive binding of precursor proteins to translocation sites in the cytoplasmic membrane SecA is needed. SecA, a protein of which cytoplasmic, peripheral as well as integral membrane forms have been detected, has an ATPase activity which mediates the initial channelling of  
10 precursor proteins into the export pathway.

The SecA subunit acts as a receptor recognizing the leader and mature domains of the preproteins (Lill et al. 1990) as well as the SecB chaperone (Hartl et al. 1990). It has been suggested that SecA penetrates into the membrane,  
15 after binding of ATP, and so promotes the coinserction of the preprotein. After hydrolysis of bound ATP the preprotein is released from the SecA protein (Schiebel et al. 1991). Translocation is completed with the proton motive force as the main driving force and requires members of the integral  
20 membrane part of the preprotein translocase complex like SecY, SecE and SecG (p12/Band1). SecD and SecF are also integral membrane proteins and are probably participating in the late steps of protein translocation.

For many years, the protein secretion machinery in  
25 prokaryotes has been considered to be independent from the protein secretion system found in higher eukaryotes (Luirink et al, 19920. In mammals, targeting of secretory proteins to the endoplasmic reticulum (ER) is mediated by the signal recognition particle (SRP), which is a ribonucleoprotein  
30 particle composed of one RNA molecule (SRP 7S RNA) and six polypeptides of 9, 14, 19, 54, 68 and 72 kD. The SRP proteins are associated with the RNA as either monomers (SRP19 and SRP54) or heterodimers (SRP9/14 and SRP68/72).

As soon as the signal peptide of secreted and  
35 transmembrane proteins has emerged from the ribosome, it is recognized and bound by SRP, which also has affinity for the ribosome. This association slows down the elongation of the

polypeptide chain (elongation arrest). When the complex of SRP, nascent polypeptide chain, and ribosome bind to the SRP receptor (SR or docking protein) associated with the ER membrane, the nascent polypeptide chain is displaced from  
5 SRP in a GTP-dependent reaction and protein translation is resumed.

The translocation of the polypeptide into the ER takes place co-translationally through a protein pore, the translocon (Gilmore et al. 1993). Thus, the SRP functions  
10 both as a cytosolic chaperone preventing premature folding of the preprotein by coupling translation to translocation and as a pilot to guide the preprotein to the SRP receptor complex in the membrane. The 54kD subunit of SRP (SRP54) binds to the signal peptide when it emerges from the  
15 ribosome and therefore seems to have a key function in the SRP-mediated process of protein secretion.

To day more and more data become available indicating that an SRP-mediated export pathway may also function in other organisms. Homologues of mammalian SRP components have  
20 been isolated from Yeast (Hann et al. 1989), E. coli (Bernstein et al. 1989, and Rymisch et al. 1989), Mycoplasma mycoides (Samuelsson, 1992) and Bacillus subtilis (Struck et al. 1989, and Honda et al. 1993).

So it is likely that an SRP-mediated pathway functions  
25 in prokaryotes in a separate secretory pathway or may form part of the general secretory pathway.

In E.coli members of an SRP-like secretory pathway were identified. These members are Ffh (Fifty four homologue) and a 4.5S RNA molecule which are homologous to the SRP54 and  
30 SRP 7S RNA of eukaryotic SRP (Ribes et al. 1990). It is shown that Ffh interacts specifically with the signal sequence of nascent presecretory proteins (Luirink et al. 1992). E.coli protein FtsY, which originally has been implicated in cell division (because its gene is located in  
35 an operon together with FtsE and FtsX) displays striking sequence similarity with the subunit of mammalian docking protein. Several observations suggest that FtsY is the

functional E.coli homologue of the mammalian SRP receptor (Luirink et al. 1994). Depletion of either FtsY, Ffh or the RNA component of the E.coli SRP affects the export of several secretory proteins.

5 Also in B. subtilis components of the SRP-like secretory pathway have been found. The Small Cytoplasmic RNA (scrRNA) was shown to have a functional relationship with the human SRP 7S RNA and the E.coli 4.5S RNA (Nakamura et al., 1992). The B.subtilis scrRNA is transcribed from the scr gene  
10 as a 354 nucleotide precursor which is then processed to a 271 nucleotide RNA at the 5' and 3' end (Struck et al., 1989), which is similar to its eukaryotic homologue (300 nucleotides) but much larger than the E.coli 4.5S RNA (114 nucleotides). Also the secondary structure of the scrRNA is  
15 very similar to the eukaryotic SRP 7S RNA, lacking only the domain III (Struck and Erdmann, 1990). This is in contrast to the other eubacterial SRP-like RNAs, which only fold into a single hairpin corresponding to domain IV (Poritz et al., 1988). Therefore the B.subtilis scrRNA is both in size and  
20 secondary structure an intermediate between prokaryotic and eukaryotic SRP-like RNA.

Besides the scr gene another gene encoding a SRP constituent has been isolated from B.subtilis. The ffh gene was found to encode the Ffh protein which shows homology to  
25 both the E.coli and eukaryotic SRP54 protein (Honda et al., 1993).

It is not unlikely that chaperones or members of the SRP-like secretion pathway may become a rate-limiting step in the secretion pathway, the result of which being that the  
30 majority of the heterologous protein expressed will aggregate or fold prematurely. This effect could be the reason why attempts to secrete heterologous proteins in high amounts from Gram-positive micro-organisms, in particular  
B.subtilis and (closely) related micro-organisms have met  
35 with little success. Overexpression of particular members of the B.subtilis secretion machinery, especially of chaperone-like proteins which are the rate-limiting step in the

secretion pathway would solve this problem. It is to be understood that the terms "chaperone" and "secretion factor" are not completely clearly defined. Both groups of proteins will at least overlap and in some cases may be identical.

- 5 Because the mechanism of action of these proteins is not yet clearly understood, both terms will be used interchangeably herein.

The invention thus provides a proteinaceous substance comprising at least a functional part of a chaperone-like protein expressed by Gram-positive bacteria encoded by the ftsY gene of said bacteria, a representative of said gene being defined by the sequence of seq. ID no. 7.

When Gram-positive bacteria, especially *Bacillus* species and in particular *Bacillus subtilis* and its closely related organisms are provided with this proteinaceous substance, of which the functionality is defined as being able to recognize a protein of interest to be secreted and lead it into the secretory pathway, they will have an enhanced capability of secreting proteins. It is very likely that proteins to be secreted must have a signal sequence, which may be their own signal sequence, or a signal sequence of a homologous protein of the host bacteria or a signal sequence homologous to the micro-organism from which the proteinaceous substance, i.e. the secretion factor according to the invention is derived.

The protein of interest may be any protein which up until now has been considered for expression in prokaryotes, as long as it can be provided or has of its own a signal sequence which render it suitable for secretion in a Gram-positive host. Of course it must also be able to be recognized (if possibly not very efficiently) and lead into the secretory pathway by the chaperone-like proteins according to the invention. It may not be the case that the chaperone-like proteins would be capable of recognizing and leading into secretion each and every protein by itself. Other secretion factors may become the rate-limiting step, if the presently invented secretion factor is provided in



sufficient quantities. In that case it is preferred to also provide the hosts with the secretion factors which may become the rate-limiting step in sufficient quantities also. Since we believe that there is a SRP-like route in *Bacillus* species and other gram-positive bacteria, it would be advantageous to provide the micro-organism with enhanced amounts of FtsY, the 7S sRNA and Ffh.

The protein of interest may be either homologous or heterologous to the host. In the first case overexpression should be read as expression above normal levels in said host. In the latter case basically any expression is of course overexpression.

The proteinaceous substance according to the invention, which for convenience will often be referred to as the chaperone, secretion factor or the chaperone-like protein, may be homologous to the host, which is preferred, but it may also be heterologous to the host, as long as it is compatible with the secretion machinery of the host. It stands to reason that this will be most likely in closely related organisms. Thus in the case of a *Bacillus subtilis* secretion factor, it would be preferred to use it in a *Bacillus*.

The sequence being depicted as giving a representative of a sequence encoding a chaperone-like protein according to the invention is given in order to enable the person skilled in the art to find homologous sequences which encode similar or functionally the same chaperone-like proteins in other Gram-positive bacteria, in particular of other *Bacillus* species. Given the general level of skill in the art, it will be routine work to prepare for instance primers based on the given sequence and to screen for other homologous sequences encoding said chaperone-like proteins. These chaperone-like proteins from other related organisms should therefore be considered as part of the present invention. Their DNA and/or amino acid sequences usually will be quite homologous. As a rule the homology will be greater than 70% overall, in particular homologies of greater than 85%.

overall are to be expected. It is understood that all homologous genes which can hybridize with the sequence depicted in seq. ID no. 7 and which encode a protein of essentially the same structure or function are comprised in this invention. The following equation, which has been derived from analyzing the influence of different factors on hybrid stability:

$$T_m = 81 + 16.6 (\log_{10} C_i) + 0.4 (\% G + C) - 600/n - 1.5\%$$
 mismatch (Ausubel et al., supra) where

$n$  = length of the shortest chain of the probe

$C_i$  = ionic strength (M)

$G + C$  = base composition,

can be used to determine which level of homology can be detected using DNA-DNA hybridisation techniques.

Therefore the term "essentially of a structure" is intended to embrace sequences which can include conservative mutations, where the sequence encodes the same amino acid, but which may differ by up to 35% in the DNA sequence according to the above equation, more typically by up to 10%. It is not always necessary to have a complete chaperone-like protein to perform the functions of recognizing the protein to be secreted and leading said protein into the secretion pathway. Where possible the hosts may thus also be provided with functional parts of said secretion factor. It is also possible and even likely that association with non-protein material such as the 7S RNA may occur when the secretion factor performs its functions. The term proteinaceous substance is chosen to include such associations. It is by now well known in the art that mutations in proteins may lead to higher activity, longer half-lives, better stability of the mutated protein. Such derivatives of the secretion factors according to the invention are also part thereof, since given the information presented herein, it is routine work to find weak spots, or other sites interesting for mutation in the secretion

factors according to the invention and making site-directed mutations.

A preferred embodiment of the present invention is a proteinaceous substance which is a chaperone-like protein which is at least partly encoded by the ftsY gene of a Bacillus species. Bacillus species are highly preferred organisms to express genes of interest in and a lot of developmental and production experience is available. As stated before however, there has always been a problem with secretion of especially heterologous proteins from Bacillus. This problem may in many cases be solved by providing Bacillus organisms with chaperone-like proteins from other related species, but it will be clear that chances of a good functional secretion factor in Bacillus, including recognition of the heterologous protein are highest using a chaperone-like protein which is derived from a secretion factor in a Bacillus species. Of special interest as a chaperonelike protein is a proteinaceous substance which is at least partly encoded by the ftsY gene of Bacillus subtilis or another Bacillus species. These proteinaceous substances are very likely to be analogues of the eukaryotic docking protein (or SRP receptor) as is the case with products derived from the E.coli ftsY gene. Lack of sufficient amounts of this chaperone-like protein will definitely have a great influence on the capability of host micro-organisms to secrete any proteins, let alone heterologous proteins. At present we believe that heterologous protein may be predominantly secreted using the SRP-like route, i.e. by binding to Ffh, the 7s RNA and the FtsY, whereas homologous proteins use the general secretion pathway. It is also clear that if a heterologous protein to be secreted is provided with a signal homologous or very closely related to a signal present in Bacillus secretory proteins that the presence of a sufficient amount of the secretion factor which normally has the function of recognizing such a signal will lead to enhanced secretion.

The preferred method of providing a Gram-positive bacteria, in particular a *Bacillus* species with the possibility of expressing sufficient amounts of the chaperone-like proteins (and for instance scRNA) according to the invention is of course by providing said micro-organism with the genetic information to overexpress said chaperone-like protein. The invention therefor also provides a recombinant DNA molecule comprising at least a part of an ftsY gene encoding a chaperone-like protein of Gram-positive bacteria, said part encoding at least a functional part of said chaperone-like protein, whereby a representative of said gene has the sequence of seq. ID no. 7.

As is true for the proteinaceous substances of the invention the given sequence is given for the reason of enabling the skilled person to find homologous sequences encoding similar secretion factors. Variants may exist within *Bacillus* species and other Gram-positive bacteria. It will also enable the person skilled in the art to construe silent mutations, to construe beneficial mutations or mutations having no effect on the activity of the chaperone resulting from expression. For different species codon preference may be different, degeneracy may be accounted for. All these modifications should be considered to be within the scope of the present invention. To define which genes still belong to the invention can really only be done by their functionality. If they encode a substance which has the same activity (in kind, not in amount) as the presently invented chaperone-like proteins then the gene (or the recombinant DNA molecule) should be considered to belong to the present invention, if the molecule is derived from a Gram-positive bacteria, in particular a *Bacillus* species. Usually this will coincide with a rather high degree of homology for instance of 70-95% overall.

A further preferred embodiment of the present invention is of course a gene or a recombinant DNA molecule comprising at least a part of the ftsY gene of a *Bacillus* species. The main reason for this preference is of course that *Bacillus*

species are well known production organisms in which for reasons already mentioned it would be helpful to provide an autologous (sometimes also called homologous) chaperone-like protein. The most preferred chaperone-like protein at the present time is the one encoded by a recombinant DNA molecule comprising at least a part of the ftsY gene of Bacillus subtilis.

For easy transfer of the genetic information of the secretion factors according to the invention it is preferred to provide the recombinant DNA molecule as a vector. The invention thus also provides a recombinant vector comprising a recombinant DNA molecule as disclosed above and suitable regulatory elements for replication and/or expression. The nature and kind of such a vector is not important, as long as it is capable of transferring the wanted genetic information into the desired micro-organism and preferably being capable of replicating or being replicated in such micro-organism. They may comprise many additional advantageous features such as marker genes, restriction sites, etc. Chromosomal integration of (part of) the gene according to the secretion factor is also comprised within this invention. It would of course be advantageous to only have to transfer a micro-organism with one vector. Preferably the invention provides a recombinant vector as described above further comprising a gene encoding a protein of interest to be secreted.

The invention further provides micro-organisms which have been provided with the genetic information to encode a chaperone-like according to the invention by whatever method. The invention thus includes a cell derived from a Gram-positive host cell comprising a recombinant DNA molecule or a vector as defined herein before. Preferably the cell is derived from a *Bacillus* species. In a further preferred embodiment the cell has also been provided with the ability to overexpress either or both 7S scRNA and Ffh in a similar manner as it has been provided with the (over)expression of FtsY.

In a further preferred embodiment the cell also has been provided with the ability to overexpress a homologous protein or to express a heterologous protein. A suitable way to arrive at such a cell is providing it with the genetic information for said protein of interest, leading to a cell comprising a vector having the genetic information encoding a chaperone-like protein according to the invention, further comprising a vector comprising a gene encoding a protein of interest to be secreted. All methods leading to the products of the invention are of course also part of this invention. In particular important are the methods leading to the enhanced production of proteins secreted in the culture medium. The invention thus also includes a method for enhancing the secretion of a protein of interest from a Gram-positive micro-organism, comprising the steps of providing said micro-organism with the possibility to overexpress the protein of interest, providing the micro-organism with the possibility of overexpressing a proteinaceous substance according to the invention, and culturing said micro-organism under suitable conditions.

Preferably the possibility to overexpress the protein of interest is provided by a vector as disclosed herein before and the possibility to overexpress a proteinaceous substance according to the invention is also provided by a vector as disclosed hereinabove.

The invention will now be further illustrated in the following detailed description and the examples.

## DETAILED DESCRIPTION OF THE INVENTION

There is now growing evidence that poor expression and/or secretion is caused by incorrect folding of the heterologous protein in the host cell. The cause of this effect may be the incompatibility of the host cell's chaperone-like proteins of the regular secretory pathway and the heterologous protein. As a result the newly synthesized heterologous proteins will be recognized very inefficiently and in this way become a rate-limiting step in the translocation process. This will be even more pronounced if the heterologous protein is overexpressed. One possibility to overcome this problem is to express heterologous chaperone-like proteins which are homologous to the heterologous protein which is to be secreted. Expression of E.coli SecB in B.subtilis has shown to facilitate secretion of the SecB-dependent maltose-binding protein of E.coli (Collier, 1994). This option is probable not applicable when the heterologous protein and secretion factor are from a more phylogenetic distant organism. In this way the host cell's regular secretion machinery could become incompatible with the heterologous chaperone-like protein itself, leaving the same effect: extreme low secretion efficiency. Another possibility, part of this invention, is to overexpress one or more of the host cell's chaperone-like proteins, preferably the SRP-like chaperone-like proteins and so increase the availability of these chaperone-like proteins for the heterologous protein.

Because homologues of SecA (Sadaie et al. 1991), SecE (Jeong et al. 1993), SecY (Su et al. 1990), and Lep (Van Dijl et al. 1992) have been identified in B.subtilis, it is suggested that signal peptide-dependent protein secretion in B.subtilis utilizes a Sec-pathway that is similar to that of E.coli. So far SecB, which is considered to be the major chaperone in E.coli, seems to be the only chaperone which has a direct binding affinity for SecA and so contributes to the accurate targeting of the preprotein-SecB complex to the membrane bound translocase. The SecB protein is needed for

only a subset of the envelope proteins so SecB independent proteins will enter the Sec-pathway with the aid of helper proteins like GroEL/GroES, DnaK/DnaJ or other proteins like SRP. In eukaryotic organism SRP mainly is responsible for the translocation across the ER membrane. Recently more evidence has become available of the existence of an SRP mediated secretion route in bacteria. Because the eukaryotic pathway has probably evolved from the bacteria it is thinkable that said proteins are also dependent on this pathway when said proteins are expressed in bacteria like *Bacillus*. Thus optimisation of this particularly pathway in *Bacillus* will be more profitable for heterologous (eukaryotic) proteins secretion than the optimisation of the well known sec-pathway. This invention relates to the cloning of the *Bacillus ftsY* gene and its effect after (over)-expression, alone or in combination with other members of the bacterial SRP, upon heterologous proteins.

For the cloning of *B. subtilis ftsY* degenerate primers were synthesized making use of the existing homology boxes between the SRP homologues of different organism (Fig. 2a). After an inversed PCR reaction using a 110 bp fragment, which was derived from a nested PCR reaction, as template a 4 kb fragment could be detected. Sequencing results (Fig. 2b) revealed an open reading frame of 329 amino acids. This protein shared 48.2% amino acid identity and 65% similarity with the *ftsY* gene of *E. coli*.

As will be shown in the Examples, the hybridizing experiments originally lead to an unwanted result, i.e. a smear of indistinct bands. Surprisingly, we were able by cutting out a region around the expected size of the amplified fragment and applying PCR to that region again in resolving this smear into a group of distinct bands. Unfortunately hardly any band was seen at the expected size of the fragment that should have been amplified. However, in a third round of amplification we were nevertheless able to obtain a fragment which could be used further.



## EXAMPLE 1

## Construction of a Promoter Vector for Secretion factor Overexpression

pHB201 is capable of autonomous replication in both  
5 E.coli (high-copy) and Bacillus (low-copy) strains. This  
plasmid confers resistance to the antibiotics  
chloramphenicol and erythromycin in E.coli and Bacillus.  
Further the plasmid carries a CAT86::lacZ fusion gene  
preceded by the strong Lactococcus lactis promoter 59 which  
10 also act as strong promoter in B.subtilis (Van der Vossen et  
al. 1987).

By replacing the SalI/EcoRI fragment of this plasmid by  
a synthetic DNA fragment (SEQ ID NO:1) the CAT86::lacZ was  
deleted and an unique NdeI restriction site introduced  
15 overlapping the translation initiation site generating  
pHBNde (Fig. 1a). This allows us to express the chaperone  
genes directly downstream the strong Lactococcal promoter 59  
without creating fusion proteins as would be with the  
original pHB201 vector (Fig. 1b).

## EXAMPLE 2

Molecular Cloning of Bacillus subtilis DNA Fragments  
Homologous With the Human SR $\alpha$  Gene

5 A set of three Polymerase Chain Reactions (PCR) were  
performed as follows. Chromosomal DNA of B. subtilis 168 was  
used as template in a first PCR reaction with degenerate  
primers AB4229 (SEQ ID NO: 2) and AB4230 (SEQ ID NO: 3).  
After 30 cycles and an annealing temperature of 40°C the  
amplified DNA was fractionised by electrophoresis on a 2%  
10 Metaphor (FMC BioProducts) agarose gel. The results showed a  
smear of ill resolved bands. DNA fragments ranging in size  
from 220 bp to 300 bp were purified from the agarose gel  
with a QIAquick gel extraction column (QIAGEN).

1/50 of these isolated fragments were used in a second  
15 PCR with degenerate primers AB4229 and AB 4241 (SEQ ID NO:  
4) using the same reaction conditions as in the first PCR.  
The result of this PCR showed a number of distinct bands,  
however, a band of the expected size ( $\pm$  120 bp) was hardly  
visible. Fragments of  $\pm$  120 bp were isolated from the  
20 agarose gel as above and used in a third PCR with the same  
primers and conditions as were used for the second PCR. The  
resulting single fragment was isolated, purified and after  
treatment with T4 polynucleotide kinase ligated into  
dephosphorylated pUC18 linearized with SmaI.

25 After electroporation to E. coli JM109, selection on  
IPTG/X-gal plates, the DNA from six white colonies were used  
for automated sequencing.

Within all six isolates an Open Reading Frame (ORF) could be  
detected. This ORF showed over 70% similarity with an  
30 alignment of homologs from several other organisms (Fig.  
2b).

## EXAMPLE 3

## Sequencing of Unknown DNA Sequences Adjacent to a Short Stretch of Known Sequence

By using a labelled internal ftsY fragment derived from example 2 we could detect a single 4 kb PstI band in a hybridisation experiment. None of the attempts to clone this fragment directly into pUC were successful, indicating that cloning of this fragment could be lethal in E.coli. An inversed PCR (IPCR) was used for determination of the sequence.

A total PstI digest of B.subtilis 168 chromosomal DNA was used as template in the IPCR with primers AB5356 (SEQ ID NO: 5) and AB5357 (SEQ ID NO: 6). The resulting fragment was used directly for automated sequencing making use of the same primers.

The sequence of the rest of the PstI chromosomal DNA fragment located upstream of the primer AB5357 and downstream of primer AB5357 was determined by automated sequencing making use of newly developed primers while the sequence was unveiled. The total 4370 bp DNA sequence of the PstI fragment is shown in Figure 3.

Analysis of the sequence showed the presence of several Open Reading Frames (ORFs), including the one for FtsY. When comparing the overall structure of the SR $\alpha$  like proteins and SRP54-like proteins a common domain is evident which comprises GTP binding boxes (the G-domain, see Figure 4). Also from this figure it is clear that the B.subtilis FtsY protein contains only a very short N-terminal domain, in contrast to the eukaryotic and E.coli homologues. Since the N-terminal domain in those organisms serves as a membrane anchor it is possible that in B.subtilis FtsY functions with a different mechanism, and is possibly more chaperone-like in its action, although it may still be membrane-bound.

Analysis of the sequence showed the presence of several more ORF's, including a truncated ORF showing homology to the Ffh protein, and a truncated ORF showing homology to

several DNA segregation proteins like the Yeast protein SMC1.

The chromosomal organisation of the genes in the PstI fragment is shown in Figure 5.

5

#### EXAMPLE 4

#### Effect of FtsY Depletion Upon the Secretion of Heterologous Proteins

The effects of depletion of FtsY in *Bacillus* on the processing and/or secretion of heterologous proteins were studied by placing the chromosomal ftsY gene under control of the inducible SPAC promoter (Yansura et. al). For this the N-terminal part of the ftsY gene was cloned into the multiple cloning site of pDG148 directly downstream the SPAC promoter. The SPAC-ftsY-penP-lacI fragment from the resulting pDGftsY' plasmid was recloned into pPPNeo2 making the final integration construct pNSftsY' (Fig. 6).

pNSftsY' is capable of autonomous replication in *E.coli* but not in *Bacillus*. It confers resistance to the antibiotic ampicillin which can be used for selection in *E.coli* and neomycin for selection in *Bacillus*. Integration of pNSftsY' into the ftsY locus results in a truncated copy of ftsY (ftsY') under control of the authentic promoter and an intact copy of ftsY under control of the SPAC promoter.

Neomycin resistant pNSftsY' integrants could be selected after a protoplast transformation of *B.subtilis* 168.

Integrants growing in a medium with 0.5 mM IPTG showed growth characteristics comparable to *B.subtilis* host lacking the integrated plasmid. The growth rate of integrants did not decrease after incubation in the absence of IPTG, nor did the cell morphology.

Effects of depletion of FtsY on protein translocation were examined by fermentation experiments using hosts expressing heterologous proteins. A pUB110 like vector containing regulatory sequences of the *B.licheniformis* or *B.amyloliquefaciens*  $\alpha$ -amylase gene was used for the

35

expression of heterologous proteins. Some of the heterologous proteins were produced to slightly higher levels in cells cultured in the presence of IPTG, however in the absence of IPTG the production of the heterologous proteins was not completely abolished.

These effects were unexpected since in E.coli depletion of FtsY has a profound effect on the cell morphology and growth rate. Therefore it is possible that there is FtsY formation despite the absence of IPTG, indicating transcriptional read through. It should be possible to correct this by insertion of a strong terminator signal upstream of the SPAC promoter in the integration construct. To confirm that the absence of FtsY is hazardous to the cell attempts were made to disrupt the ftsY gene.

#### EXAMPLE 5

Construction of Bacillus strains without a functional ftsY gene.

Since the experiments described in Example 4 were unexpected in the sense that strain containing the ftsY gene under the control of the SPAC promoter were still viable, and showed no clear phenotype in the absence of the inducer IPTG, we hypothesized that the construct we used was leaky so that even in the absence of IPTG a small amount of FtsY would be produced. To eliminate the production of FtsY we tried to disrupt the ftsY gene by insertion of a neomycin resistance marker. We constructed a plasmid pBHdSFtsYNeo which harbours the 5' and the 3' end of the ftsY gene separated by the Neomycin resistance gene in a vector unable to replicate in B.subtilis.

Plasmid pBHdSFtsYNeo was linearized by cutting with HpaI and used to transform B.subtilis 168 to neomycin resistance. This should lead to strains having the original ftsY gene replaced by the ftsY::Neo construct via a double cross-over event (see Figure 7). However, we were unable to select for Neomycin resistant colonies using this linear

DNA, suggesting the possibility that disruption of the ftsY gene is lethal to the cell.

We therefore repeated the transformation experiments with intact, uncut, plasmid DNA. In this case integration of the plasmid into the ftsY gene can take place via a single cross-over event (Campbell type integration), leading to neomycin resistant colonies which have both an intact and a disrupted copy of the ftsY gene present (see Figure 8). We isolated integrant strains, one of which was shown to have the chromosomal organization represented in Figure 8. Since in this strain two copies of the 3' end of the ftsY gene are present recombination between them is possible, leading to excision of the plasmid sequences in between and formation of a strain containing only one copy of the ftsY gene, disrupted by the neomycin resistance marker. Despite several attempts we were unable to isolate such recombinant strains, suggesting again that such strains are unviable.

#### EXAMPLE 6

##### Effect of FtsY Overexpression on the Location of Precursors and Mature Heterologous Proteins

The effects of overexpression of FtsY in *Bacillus* on the processing and/or secretion of heterologous proteins were studied by placing the complete ftsY gene under control of the constitutive P59 promoter in pHBNde (see Example 1) resulting in the plasmid pHBFTsY. Effects of overexpression of FtsY on protein translocation were examined by pulse-chase experiments using hosts expressing heterologous proteins.

B. Licheniformis T399 was transformed with plasmid pLAT-IL3 containing the human Interleukin-3 (h-IL-3) gene expressed from the B. licheniformis  $\alpha$ -amylase promoter and provided with the B. licheniformis  $\alpha$ -amylase signal sequence, or with the plasmid pLP10-AB containing the prochymosin gene under the same expression signals. The resulting strains T399IL and T399Chy were transformed with plasmid pHBNFTsY

containing the *ftsY* gene. As a control also both strains T399IL and T399Chy were transformed with the vector pHBNde.

Single colonies of all strains were inoculated in 5 ml of medium starvation medium S7+ (including Methionine and Cysteine) and grown at 37°C overnight. Aliquots of 200µl were inoculated in 5 ml S7- (without Methionine and Cysteine) medium and grown for another 5 hours.

After growth to OD=0.3-0.7 a sample of 3.2 ml was centrifuged, washed with S7- medium and resuspended into 3.2 ml fresh S7- medium. The sample was incubated for 20 minutes at 37°C and pulsed with 25µ(Ci L- [<sup>35</sup>S]-Methionine (>1000 Ci/mmol) per ml during 60 seconds at 37°C. Then a chase was performed by addition of 50µl (2 mg/ml) L- Methionine per ml. The chase was stopped at different time points (0, 15, 30, and 60 seconds) by mixing of 600µl of the reaction with 600µl ice cold 20% TCA, and incubation on ice for at least 30 minutes. The samples were centrifuged, and the supernatant was used directly for immuno precipitation, SDS-polyacrylamide gel electrophoresis and autoradiography using standard protocols.

The cell pellet was washed with 1 ml acetone, and dried. The cells were resuspended in 50µl lysis buffer (10 mM Tris pH8, 25 mM MgCl<sub>2</sub>, 200 mM NaCl, 5 mg/ml lysozyme) and incubated for 3 minutes at 37°C. After addition of 50µl TES (20 mM Tris, 2 mM EDTA, 2% SDS, pH8) the samples were boiled for 5 minutes. To the samples was added 900µl of STDT (10 mM Tris, 0.9% NaCl, 1% Triton, 0.5% Sodium deoxycholate, pH8.2), the mixture was incubated for 15-60 minutes on ice, and the debris was precipitated. The supernatant was used for immuno precipitation with antiserum raised against h-IL-3 or Chymosin and subsequent SDS-polyacrylamide gel electrophoresis and autoradiography using standard protocols.

Overexpression of FtsY increased the secretion of the mature form of interleukin-3 and the processing of the precursor of prochymosin.

## EXAMPLE 7

Effect of Overexpression of Ffh on the Secretion of Human Interleukin-3.

- The ffh gene encoding the B. subtilis homologue of the eukaryotic SRP54 protein was cloned as a PCR fragment obtained using primers based on the published DNA sequence (Honda et al, 1993). The gene was cloned into the vector pHBNde (see Example 1) under the control of the strong Lactococcal P59 promoter to form plasmid pHBNFfh.
- Pulse-chase experiments were performed as described in Example 6 with B. licheniformis strains containing both plasmid pHBNffh and plasmid pLAT-IL3 harbouring the human Interleukin-3 (h-IL-3) gene under the expression and secretion signals of the B. licheniformis  $\alpha$ -amylase gene.
- As is shown in Figure 9, the processing of h-IL-3 is very fast, as no precursor can be detected. The amount of mature h-IL-3 in the supernatant fraction is in all cases higher in the samples obtained from the strain overproducing Ffh compared to the strain containing only the vector plasmid.

## EXAMPLE 8

Effects of Overexpression of scrRNA on the secretion of Human Interleukin-3.

- The scr gene encoding the scrRNA from B. subtilis was cloned as a PCR fragment using primers based on the published DNA sequence (Struck et al, 1989) and following the approach (including the same 5' primer) described by Nakamura (Nakamura et al, 1992) introducing a HindIII site just upstream of the scr DNA and a SphI site downstream of the terminator sequence.

- The P59 promoter was deleted from the vector pHB210 by replacement of the AlwNI-SmaI fragment containing the origin of replication (ori) together with the P59 promoter by the AlwNI-PvuII fragment from vector pBR322 containing only the same ori. This new vector pBHK was used to exchange the EcoRI-PvuII fragment for the EcoRI-BamHI



(blunted by T4 polymerase) containing the SPAC-penP-lacI cassette from pDG148 to construct the vector pBHSpac.

The PCR fragment containing the scr gene was digested with HindIII and SphI and ligated into pBHSpac digested with  
5 the same restriction enzymes to construct plasmid pBHSScr. This way the scr gene was placed under the control of the SPAC promoter.

Pulse-chase experiments were performed as described in Example 6 with B.licheniformis strains containing both  
10 plasmid pBHSScr and plasmid pLAT-IL3 harbouring the human Interleukin-3 (h-IL-3) gene under the expression and secretion signals of the B.licheniformis  $\alpha$ -amylase gene.

Also in this case the processing of h-IL-3 was very fast, as no precursor could be detected. However, the amount  
15 of mature h-IL-3 in the supernatant fraction was in all cases higher in the samples obtained from the strain overproducing scrRNA compared to the strain containing only the vector plasmid, or with plasmid pBHSScr in the absence of the inducer IPTG.

20

#### EXAMPLE 9

Effects of Simultaneously Overexpressed Signal Recognition Particle components on the Secretion of Heterologous Proteins.

25 The effects described in Examples 6, 7 and 8 were even more pronounced when more than one of the components of the bacterial signal recognition particle were simultaneously expressed. For this purpose the ftsY gene was expressed from the IPTG inducible promoter located in the chromosome as  
30 described in Example 4 by addition of 3 mM IPTG, while the components Ffh or scrRNA were expressed from their pHB210 derived vectors described in Examples 7 and 8 above.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Genencor International B.V.

(B) STREET: Wateringseweg 1

(C) CITY: Delft

(E) COUNTRY: The Netherlands

(F) POSTAL CODE (ZIP): 2600 MA

(ii) TITLE OF INVENTION: Secretion of heterologous proteins in  
Bacillus

(iii) NUMBER OF SEQUENCES: 8

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 170 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: AB4296-4303

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCTCTAGAGT AGATCTGCAG GCTTTAACGT AGGCAAAGCT CAGGGTAGAC TTTGAATGGA 60

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TCCTGAATAC AGTAAATCAC ATTCAGGAGG AGATACATAT GGAATTCGTA 170

## (2) INFORMATION FOR SEQ ID NO: 2:

26

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: AB4229

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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20

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: AB4230

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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## (2) INFORMATION FOR SEQ ID NO: 4:

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- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

27

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: AB4241

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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17

(2) INFORMATION FOR SEQ ID NO: 5:

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(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB5356

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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34

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB5357

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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34

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1016 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *B. subtilis*

(B) STRAIN: 168

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 19..1008

(D) OTHER INFORMATION: /function= "protein secretion  
chaperone"

/product= "FtsY"

/gene= "ftsY"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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Met Ser Phe Phe Lys Lys Leu Lys Glu Lys Ile

1 5 10

ACA AAA CAG ACA GAT TCC GTA TCT GAA AAG TTT AAG GAT GGC CTT GAA 99

Thr Lys Gln Thr Asp Ser Val Ser Glu Lys Phe Lys Asp Gly Leu Glu

15 20 25

AAA ACA AGA AAC TCC TTT CAA AAC AAA GTG AAT GAT CTT GTA TCC CGT 147

Lys Thr Arg Asn Ser Phe Gln Asn Lys Val Asn Asp Leu Val Ser Arg

30 35 40

TAC CGT AAA GTG GAT GAG GAT TTC TTC GAA GAG CTT GAA GAG GTT CTT 195

Tyr Arg Lys Val Asp Glu Asp Phe Phe Glu Glu Leu Glu Glu Val Leu

45 50 55

ATC AGC GCG GAT GTC GGT TTT ACA ACC GTT ATG GAA TTA ATA GAT GAG 243  
 Ile Ser Ala Asp Val Gly Phe Thr Thr Val Met Glu Leu Ile Asp Glu  
 60 65 70 75

CTG AAA AAA GAA GTC AAA CGC AGA AAT ATT CAA GAT CCA AAG GAA GTC 291  
 Leu Lys Lys Glu Val Lys Arg Arg Asn Ile Gln Asp Pro Lys Glu Val  
 80 85 90

AAG TCA GTG ATT TCT GAG AAA CTG GTC GAG ATT TAT AAC AGC GGA GAT 339  
 Lys Ser Val Ile Ser Glu Lys Leu Val Glu Ile Tyr Asn Ser Gly Asp  
 95 100 105

GAG CAA ATT TCA GAA CTG AAC ATC CAG GAT GGG CGT TTA AAC GTA ATC 387  
 Glu Gln Ile Ser Glu Leu Asn Ile Gln Asp Gly Arg Leu Asn Val Ile  
 110 115 120

CTT CTG GTA GGT GTA AAC GGC GTC GGG AAA ACA ACA ACG ATC GGA AAG 435  
 Leu Leu Val Gly Val Asn Gly Val Gly Lys Thr Thr Thr Ile Gly Lys  
 125 130 135

CTT GCT CAT AAA ATG AAA CAA GAA GGA AAA TCT GTT GTA CTT GCC GCC 483  
 Leu Ala His Lys Met Lys Gln Glu Gly Lys Ser Val Val Leu Ala Ala  
 140 145 150 155

GGA GAC ACT TTT AGA GCG GGA GCC ATT GAA CAG CTG GAA GTA TGG GGA 531  
 Gly Asp Thr Phe Arg Ala Gly Ala Ile Glu Gln Leu Glu Val Trp Gly  
 160 165 170

GAG CGT ACA GGA GTG CCT GTC ATT AAG CAG ACG GCA GGA AGC GAT CCG 579  
 Glu Arg Thr Gly Val Pro Val Ile Lys Gln Thr Ala Gly Ser Asp Pro  
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GCG GCT GTC ATC TAC GAT GCT GTT CAT GCT GCG AAA GCA AGA AAT GCC 627  
 Ala Ala Val Ile Tyr Asp Ala Val His Ala Ala Lys Ala Arg Asn Ala  
 190 195 200

GAT GTA TTA ATT TGT GAT ACG GCA GGG CGT CTC CAA AAC AAA GTA AAT 675  
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 205 210 215

CTC ATG AAA GAG CTT GAA AAA GTA AAA CGT GTT ATC GAA AGA GAA GTT 723  
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 220 225 230 235

CCT GAA GCT CCG CAT GAG GTG CTG CTT GCC CTT GAT GCC ACG ACC GGC 771  
 Pro Glu Ala Pro His Glu Val Leu Leu Ala Leu Asp Ala Thr Thr Gly  
 240 245 250

CAA AAT GCA ATG GCT CAG GCA AAA GAA TTC TCT AAA GCA ACA AAT GTT 819  
 Gln Asn Ala Met Ala Gln Ala Lys Glu Phe Ser Lys Ala Thr Asn Val  
 255 260 265



ACC GGC ATT GCT TTA ACG AAG CTT GAC GGT ACG GCA AAA GGC GGT ATC 867  
 Thr Gly Ile Ala Leu Thr Lys Leu Asp Gly Thr Ala Lys Gly Gly Ile  
 270 275 280

GTC CTT GCG ATT CGC AAC GAG CTT CAC ATC CCG GTT AAA CTA GTC GGT 915  
 Val Leu Ala Ile Arg Asn Glu Leu His Ile Pro Val Lys Leu Val Gly  
 285 290 295

TTA GGA GAA AAA GTT GAT GAC CTT CAG GAA TTT GAT CCA GAA TCC TAT 963  
 Leu Gly Glu Lys Val Asp Asp Leu Gln Glu Phe Asp Pro Glu Ser Tyr  
 300 305 310 315

GTG TAC GGA CTC TTT TCA GAT TTA GTG GAA AAA GCC GAC GAT TAAGAAAAAG 1015  
 Val Tyr Gly Leu Phe Ser Asp Leu Val Glu Lys Ala Asp Asp  
 320 325 330

G 1016

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 329 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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 1 5 10 15

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 20 25 30

Phe Gln Asn Lys Val Asn Asp Leu Val Ser Arg Tyr Arg Lys Val Asp  
 35 40 45

Glu Asp Phe Phe Glu Glu Leu Glu Glu Val Leu Ile Ser Ala Asp Val  
 50 55 60

Gly Phe Thr Thr Val Met Glu Leu Ile Asp Glu Leu Lys Lys Glu Val  
 65 70 75 80

Lys Arg Arg Asn Ile Gln Asp Pro Lys Glu Val Lys Ser Val Ile Ser  
 85 90 95

Glu Lys Leu Val Glu Ile Tyr Asn Ser Gly Asp Glu Gln Ile Ser Glu  
 100 105 110

Leu Asn Ile Gln Asp Gly Arg Leu Asn Val Ile Leu Leu Val Gly Val  
115 120 125

Asn Gly Val Gly Lys Thr Thr Thr Ile Gly Lys Leu Ala His Lys Met  
130 135 140

Lys Gln Glu Gly Lys Ser Val Val Leu Ala Ala Gly Asp Thr Phe Arg  
145 150 155 160

Ala Gly Ala Ile Glu Gln Leu Glu Val Trp Gly Glu Arg Thr Gly Val  
165 170 175

Pro Val Ile Lys Gln Thr Ala Gly Ser Asp Pro Ala Ala Val Ile Tyr  
180 185 190

Asp Ala Val His Ala Ala Lys Ala Arg Asn Ala Asp Val Leu Ile Cys  
195 200 205

Asp Thr Ala Gly Arg Leu Gln Asn Lys Val Asn Leu Met Lys Glu Leu  
210 215 220

Glu Lys Val Lys Arg Val Ile Glu Arg Glu Val Pro Glu Ala Pro His  
225 230 235 240

Glu Val Leu Leu Ala Leu Asp Ala Thr Thr Gly Gln Asn Ala Met Ala  
245 250 255

Gln Ala Lys Glu Phe Ser Lys Ala Thr Asn Val Thr Gly Ile Ala Leu  
260 265 270

Thr Lys Leu Asp Gly Thr Ala Lys Gly Gly Ile Val Leu Ala Ile Arg  
275 280 285

Asn Glu Leu His Ile Pro Val Lys Leu Val Gly Leu Gly Glu Lys Val  
290 295 300

Asp Asp Leu Gln Glu Phe Asp Pro Glu Ser Tyr Val Tyr Gly Leu Phe  
305 310 315 320

Ser Asp Leu Val Glu Lys Ala Asp Asp  
325

CLAIMS

1. A proteinaceous substance comprising at least a functional part of a secretion factor expressed by Gram-positive bacteria encoded by the *ftsY* gene of said bacteria, a representative of said gene being defined by the sequence  
5 of seq. ID no. 7.
2. A proteinaceous substance according to claim 1, which is at least partly encoded by the *ftsY* gene of a *Bacillus* species.
- 10 3. A proteinaceous substance according to claim 2, which is at least partly encoded by the *ftsY* gene of *Bacillus subtilis*.
- 15 4. A proteinaceous substance according to claim 3, which is at least partly encoded by the *ftsY* gene having the sequence of seq. ID no. 7.
- 20 5. A recombinant DNA molecule comprising at least a part of an *ftsY* gene encoding a chaperone protein of Gram-positive bacteria, said part encoding at least a functional part of said chaperone protein, whereby a representative of said gene has the sequence of seq. ID no. 7.
- 25 6. A recombinant DNA molecule according to claim 5, comprising at least a part of the *ftsY* gene of a *Bacillus* species.
7. A recombinant DNA molecule according to claim 6, comprising at least a part of the *ftsY* gene of *Bacillus subtilis*.
- 30 8. A recombinant vector comprising a recombinant DNA molecule according to anyone of claims 5-7 and suitable regulatory elements for replication and/or expression.
9. A recombinant vector according to claim 8 further

comprising a gene encoding a protein of interest to be secreted.

10. A cell derived from a Gram-positive host cell comprising

5 a recombinant DNA molecule according to anyone of claims 5-7 or a vector according to claim 8 or 9.

11. A cell according to claim 10, further comprising a vector comprising a gene encoding a protein of interest to be secreted.

10 12. A cell according to claim 10 or 11, further comprising an enhanced amount of either or both Ffh and 7S scrRNA.

13. A cell according to claim 12 whereby the enhanced amount of Ffh and/or scrRNA are the result of overexpression of the respective genes therefor.

15 14. A cell according to claim 14 whereby said overexpression is the result of the presence of at least one vector comprising a gene encoding Ffh or 7S scrRNA.

15. A method for enhancing the secretion of a protein of interest from a Gram-positive micro-organism, comprising the  
20 steps of providing said micro-organism with the capability to over express the protein of interest, providing the micro-organism with the capability of overexpressing a proteinaceous substance according to claim 1, and culturing said micro-organism under suitable conditions.

25 16. A method according to claim 15 wherein the capability to overexpress the protein of interest is provided by a vector according to claim 8 or 9.

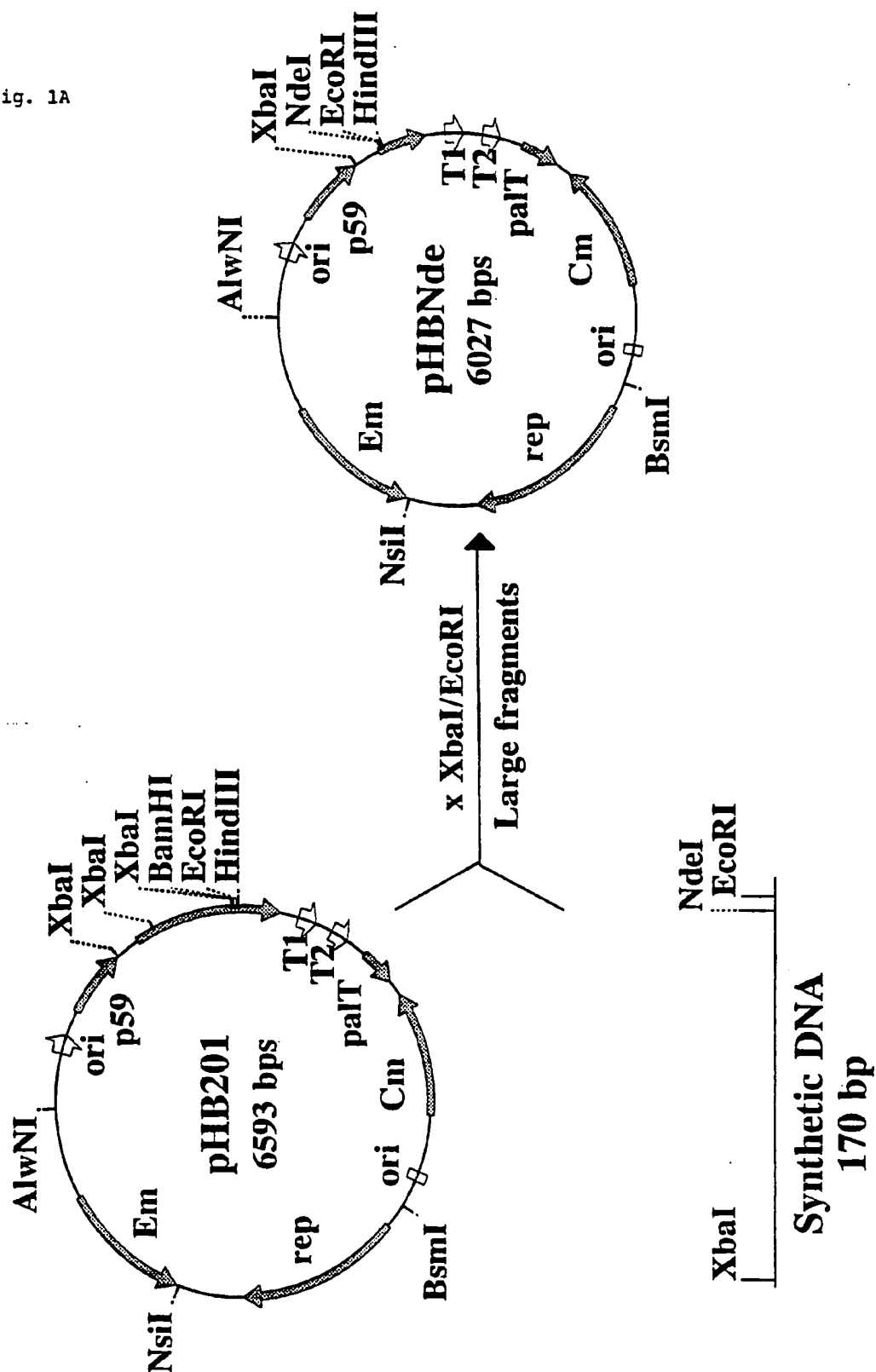
17. A method according to claim 15 wherein the capability to overexpress proteinaceous substance according to claim 1  
30 is provided by a vector according to claim 8 or 9.

18. A method according to anyone of claims 15-17, further comprising the step of providing the micro-organism with the capability of overexpressing either or both 7S scrRNA or Ffh derived from the same or a different gram-positive organism.

35 19. A method according to claims 15-18, wherein the gram-positive organism is a Bacillus species.

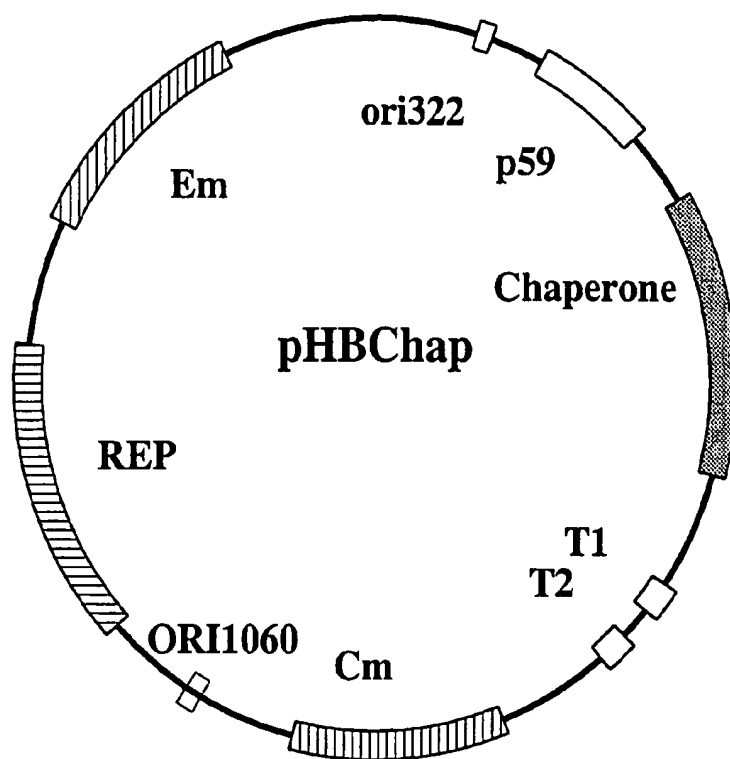
20. A method according to claim 19, wherein the *Bacillus* species is selected from the group comprising *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus alcalophilus*, *Bacillus centus* and *Bacillus*  
5 *stearothermophilus*.

Fig. 1A



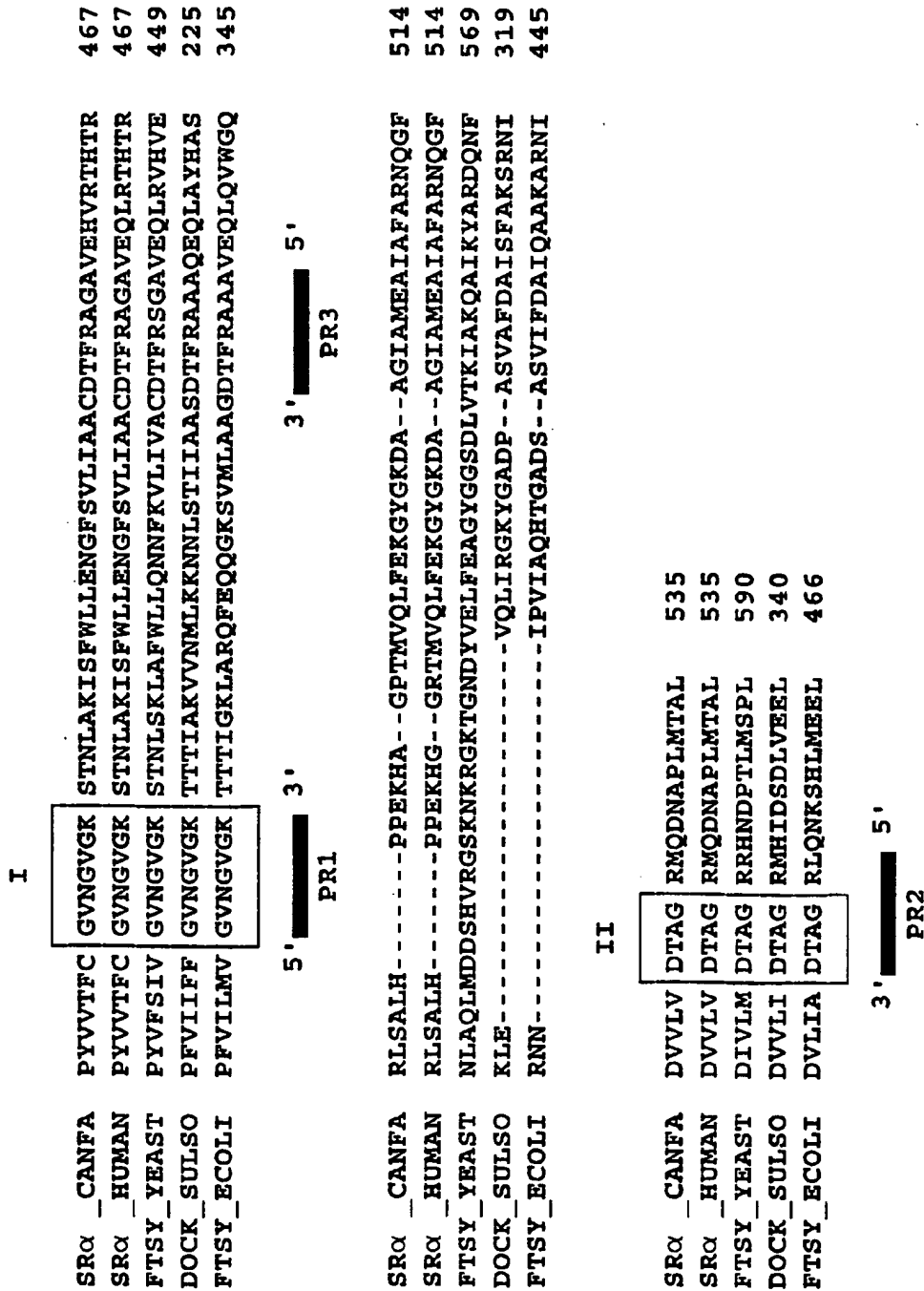
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Fig. 1B



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Fig. 2A





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Fig. 2B

SR $\alpha$ _CANFA	S	T	N	L	A	K	I	S	F	W	L	L	E	N	G	F	S	V	L	I	A	A	C	454
SR $\alpha$ _HUMAN	S	T	N	L	A	K	I	S	F	W	L	L	E	N	G	F	S	V	L	I	A	A	C	454
FTSY_YEAST	S	T	N	L	S	K	L	A	F	W	L	L	Q	N	N	F	K	V	L	I	V	A	C	433
DOCK_SULSO	T	T	I	A	K	V	V	N	M	L	K	K	Q	N	N	L	S	T	I	I	A	A	S	209
FTSY_ECOLI	T	T	I	G	K	L	A	R	Q	F	E	Q	Q	Q	G	K	S	V	M	L	A	A	G	329
	.	*	.	.	.	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*		
FTSY_BSUB	T	T	I	G	K	L	A	H	K	M	K	Q	Q	E	G	K	S	V	V	L	A	A	G	

Identity : 3 ( 13.0%)

Similarity: 14 ( 60.9%)

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## \* \* \* MOLECULE FEATURES \* \* \*

Type	Start	End	Compl Name	Description
GENE	1	2286	'orf2	'orf2 = 3' part of ORF showing homology to DNA segragation genes like Yeast SMC1.
GENE	2306	3295	FtsY	FtsY
REGION	3294	3329	T	Terminator
GENE	3673	3332 (C)	orf3	orf3
REGION	3904	3939	P	promoter orf2-ffh operon
GENE	3992	4324	orf1	orf1
GENE	4338	4370	ffh'	ffh' = 5' part of ffh gene

FIG. 3

NAME: FTSY\_BACSU\_TO 4370 BPS DNA

\* \* \* S E Q U E N C E \* \* \*

```
1  CTGCAGGAAC GGCATGATAT TTCTGCGCGT AAAGCCGCAT GTGAAACGGA ATTTGCCCGA
61  ATTGAGCAGG AGATTCACAG TCAAGTCGGT GCATATCGTG ATATGCAGAC AAAATATGAG
121 CAGAAAAAGC GCCAATACGA AAAAAATGAA TCCGCTCTGT ATCAGGCATA CCAATACGTT
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301 GTTCTTGAGC TGATTTCTAC AGAACAGAAG TATGAAACGG CCATTGAAAT AGCGCTCGGC
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421 TTAAAGCAGA ATTCCTTCGG CCGGGCGACG TTTCTGCCTC TTTCTGTTAT TAGAGACCGC
481 CAGCTTCAAA GCCGTGACGC GGAAACAGCC GCCCGGCATT CATCATTTCT CGGGGTGACC
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661 TACCGCATCG TAACCCTTGA GGGAGATGTT GTGAACCCCG GTGTTCAAT GACGGGCGGC
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1261 GAGCTTGCGT TAAAGAAGC AAAAGAAGAC TTAAGCTTCT TAACGTCAGA GATGTCATCT
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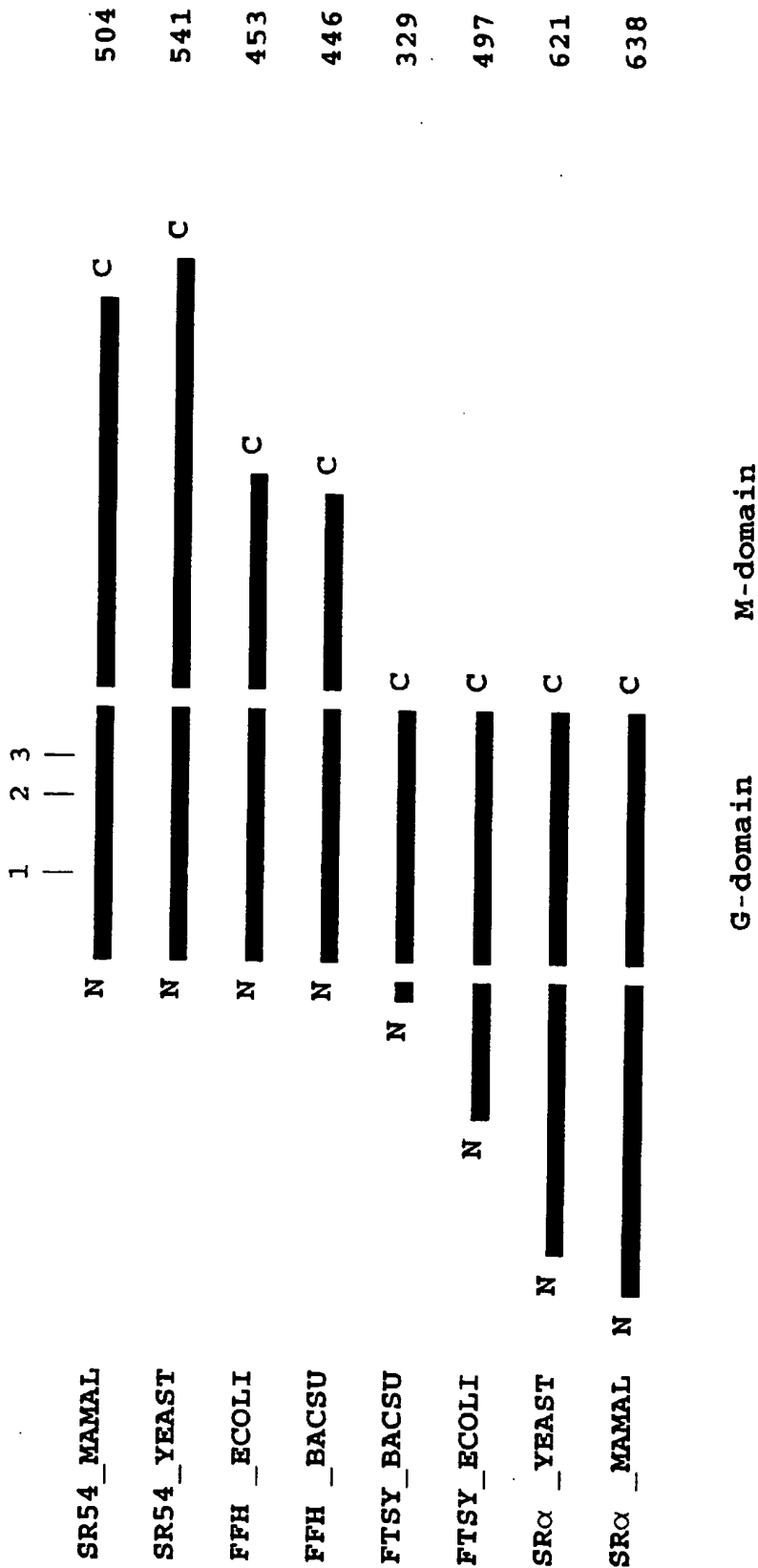
SUBSTITUTE SHEET (RULE 26)

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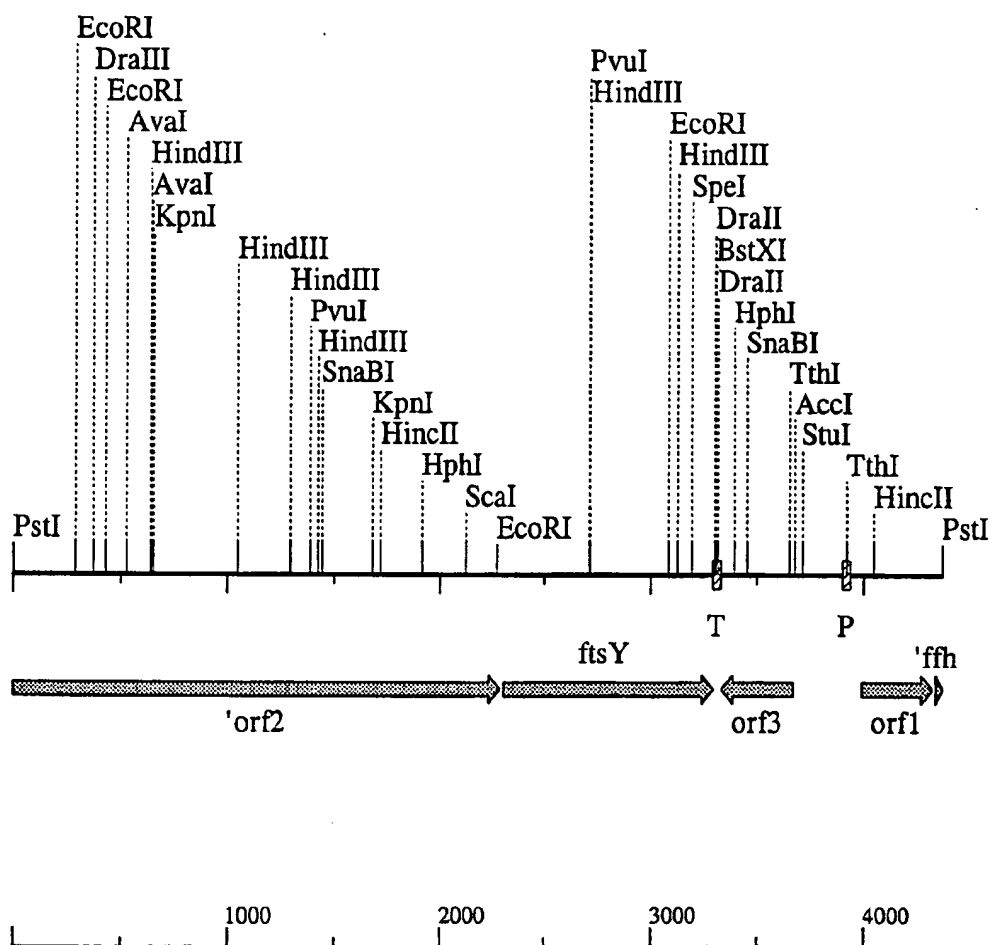
Schematic alignment of SRP54, Ffh, FtsY and SR $\alpha$

Fig. 4



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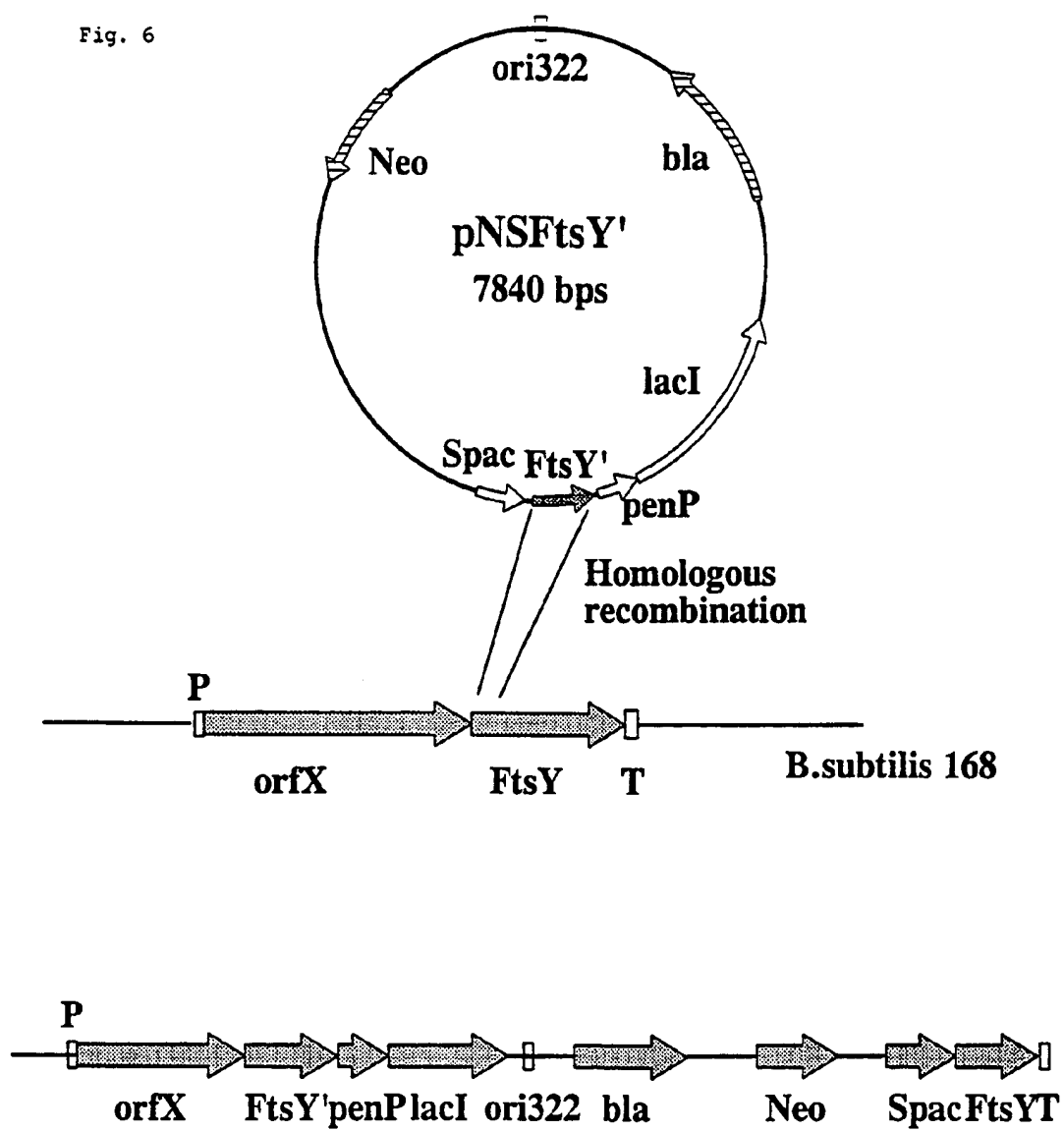
Fig. 5

**B. subtilis chromosomal ftsY region**

(4370 bps)

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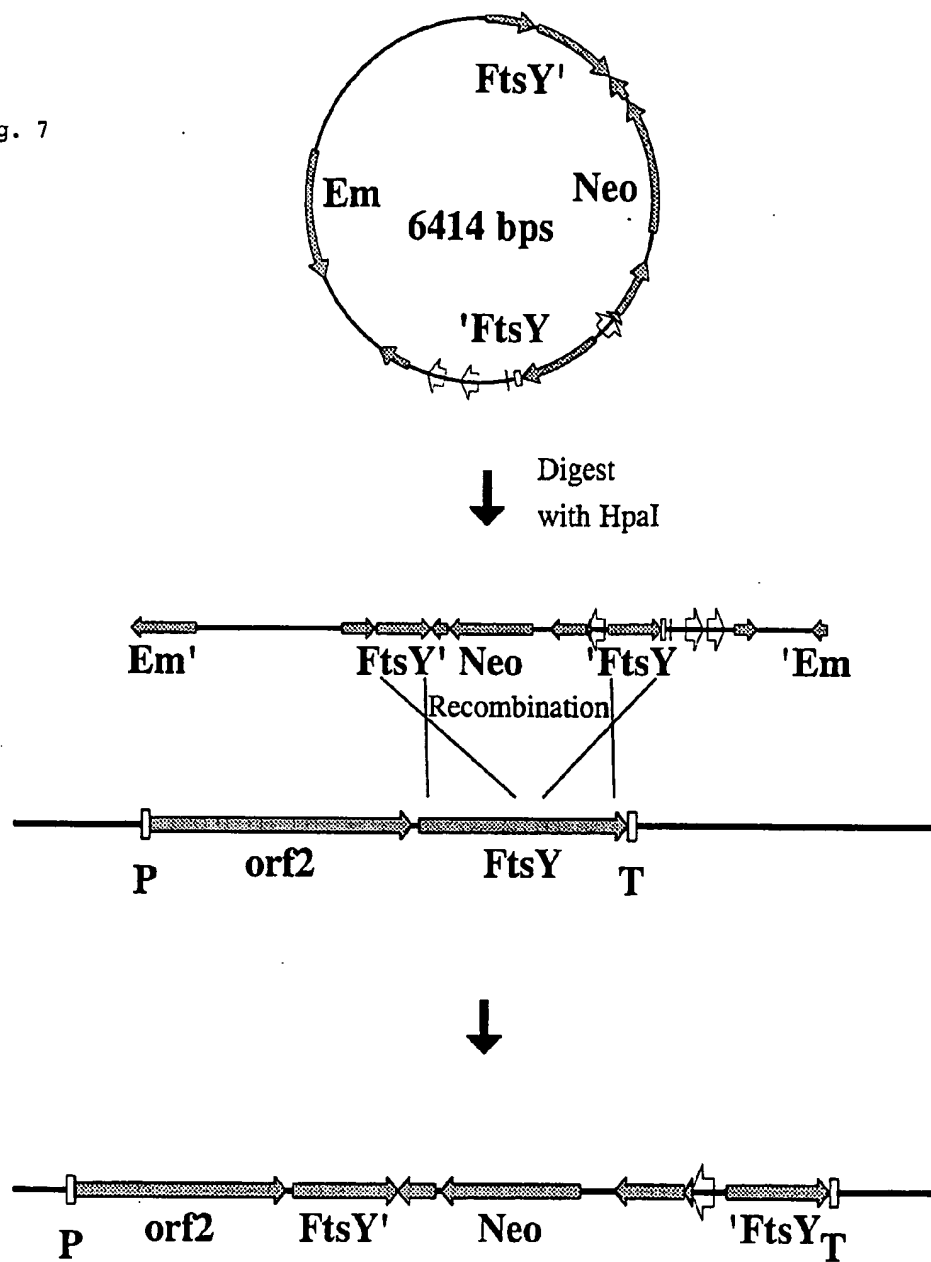
Fig. 6





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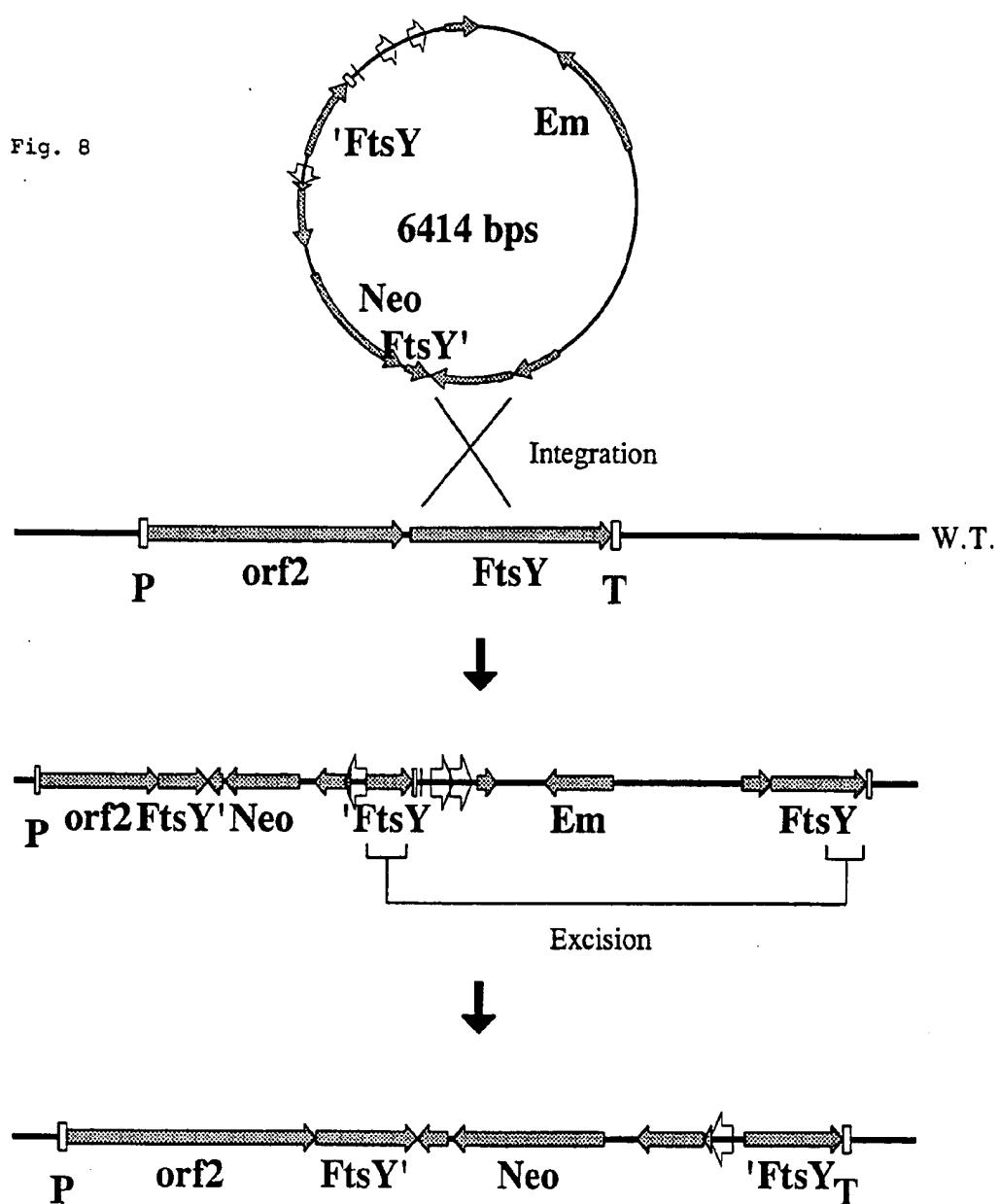
Fig. 7



Construction of *ftsY* disruption strain.

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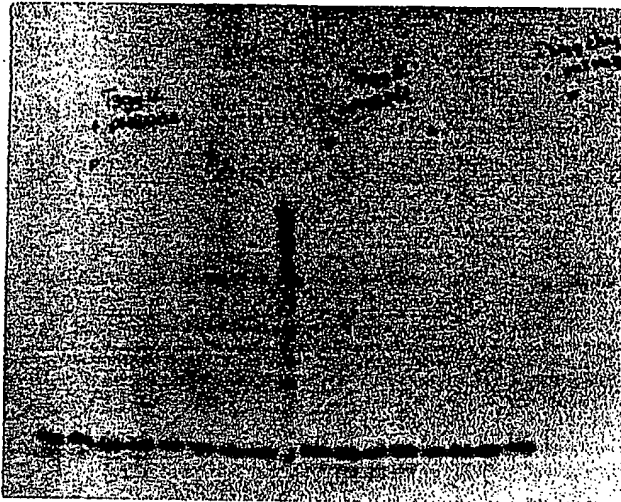
Fig. 8



Construction of *ftsY* disruption strain.

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Fig. 9



0°15'30"60" 0°15'30"60" 0°15'30"60" 0°15'30"60"  
pellet sup pellet sup

# INTERNATIONAL SEARCH REPORT

International Application No

PC1/NL 96/00278

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K14/32 C12N1/21 C12N15/67 C12N15/75  
 //(C12N1/21,C12R1:07)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. BACTERIOL., vol. 175, 1993, pages 4885-4894, XP000608463 HONDA K. ET AL.: "Cloning and characterization of a Bacillus subtilis gene encoding a homolog of the 54-kilodalton subunit of mammalian signal recognition particle and Escherichia coli Ffh" cited in the application see page 4892, right-hand column, last paragraph; figures 3,9 --- -/--	1-20

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
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- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- \* & \* document member of the same patent family

Date of the actual completion of the international search

29 October 1996

Date of mailing of the international search report

05. 11. 96

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 NL - 2280 HV Rijswijk  
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Authorized officer

Espen, J

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/NL 96/00278

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DNA RES, 1995, 2 (2) P95-100, JAPAN, XP000607148 OGURO A ET AL: "srb: a Bacillus subtilis gene encoding a homologue of the alpha-subunit of the mammalian signal recognition particle receptor." see figures 2,4 ---	1-20
Y	EMBO J, MAY 15 1994, 13 (10) P2289-96, ENGLAND, XP000608279 LUIRINK J ET AL: "An alternative protein targeting pathway in Escherichia coli: studies on the role of FtsY." cited in the application see the whole document ---	1-20
Y	NATURE, FEB 17 1994, 367 (6464) P657-9, ENGLAND, XP000608227 MILLER JD ET AL: "Interaction of E. coli Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor [see comments]" see the whole document ---	1-20
Y	CELL, 77 (6). 1994. 787-790., XP000608274 WOLIN S L: "From the elephant to E. coli: SRP-dependent protein targeting" see the whole document ---	1-20
A	QUAX W J ET AL: "Correct secretion of heterologous proteins from Bacillus licheniformis", 0 (0). 1993. 143-147. XP000607472 in 'Industrial Microorganisms: Basic and Applied Molecular Genetics', BALTZ R H ET AL (EDS) -----	